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DNA taxonomy reveals high species diversity among the stygobiont genus *Metastenasellus* (Crustacea, Isopoda) in African groundwater

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Abstract

This study aimed to explore the species diversity within the isopod genus *Metastenasellus* in Benin and Cameroon. Compared to other parts of the world, the described diversity of stygobiotic crustaceans in Africa is low due to a dearth of studies and taxonomic expertise. However, recent research activities in Benin and Cameroon suggest higher groundwater diversity than previously envisioned. Recent sampling campaigns in these countries have shown that *Metastenasellus* is a major group in the underground aquatic environment. The accumulation of biological material provided an opportunity to explore species diversity within the genus using a DNA taxonomy approach based on the cytochrome *c* oxidase subunit I (COI) gene fragment.

Despite the limitations of using a single-locus approach for species delimitation, an overview of the diversity within the genus *Metastenasellus* was obtained, revealing the presence of 23 distinct lineages. Several elements suggest that most, if not all, of these lineages represent valid species. These include high genetic distances between lineages, morphologically distinct species separated by genetic distances of the same order of magnitude as between other described lineages, and the coexistence of different lineages at the same stations.

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Despite a limited sampling effort, these first results indicate a high level of species diversity and endemism within *Metastenasellus* in the studied regions. The narrow geographic distribution of the lineages suggests strong isolation and limited dispersal abilities. This study highlights the potential for discovering a significant number of new species within this genus and emphasizes the need for further research to uncover the extent of diversity in African stygobiotic isopods.

Keywords
Africa, Diversity, DNA taxonomy, Endemism, Genus *Metastenasellus*, Groundwater Isopoda

Introduction

Obligate groundwater organisms, or stygobites, are known in all animal groups, including invertebrates and vertebrates (Botosaneanu 1986; Gunn 2004), but crustaceans constitute 43% of their total known diversity (Gibert and Culver 2009). In Africa, the Isopoda is the order that contains the largest number of stygobiotic species among the crustaceans (6 families, 23 genera, 80 species) (Tuekam Kayo et al. 2012). The asellote Stenasellidae is a major group in the groundwater (Magniez 1999) and is the most diverse family in Africa (24 species validly described to date), found mainly in tropical Africa, from Côte d'Ivoire to Kenya (Tuekam Kayo et al. 2012; Pountougnigni et al. 2021). Within the family, the genus *Metastenasellus* Magniez, 1966 includes nine species described to date with trans-Saharan distribution, with one species known from Algeria and 8 others distributed in West and Central Africa (DRC, Cameroon, Côte d'Ivoire, Nigeria) (Pountougnigni et al. 2021).

Compared to other parts of the world, information on the diversity and endemism of stygobiotic crustaceans found in Africa remain very low due to a lack of studies and a deficit in taxonomic expertise (Boutin et al. 2011; Tuekam Kayo et al. 2012). Recent years have seen the development of research activities in both Benin and Cameroon to document groundwater biodiversity in relation to water quality, vulnerability to pollution and local use (see among others Tuekam Kayo 2013; Lagnika et al. 2014; Lagnika 2015; Martin et al. 2019; Tuekam Kayo et al. 2021). First results suggest a much higher stygofaunal diversity in these countries than is currently known. The stygobiotic genus *Metastenasellus* is a good biological model to investigate this issue.

Although no species has yet been formally described in Benin, *Metastenasellus* has been observed on several occasions, during surveys of the faunistic and water quality of wells in that country (Lagnika et al. 2014; Lagnika 2015). Sequences of the cytochrome *c* oxidase subunit (COI) gene fragment are available for two *Metastenasellus* specimens from Benin (Eme et al. 2018). In Cameroon, Zébazé Togouet et al. (2009) reported for the first time two undescribed *Metastenasellus* species in the country. The genus was mentioned in several wells of Yaoundé (Tuekam Kayo 2013) and *Metastenasellus camerounensis* Zébazé Togouet, Boulanouar, Njiné & Boutin, 2013 was eventually the first species to be described in Cameroon (Zébazé Togouet et al. 2013). Later, two undescribed species were mentioned from the Bamoun Plateau in the Western Region of

Cameroon (Nana Nkemegni et al. 2015). Although initially reported as *Stenasellus* species, these two species were later treated as species of *Metastenasellus* (Pountougnigni et al. 2021), which is indeed more consistent with the disjunct distribution of these genera in Africa. First COI sequences were provided for *Metastenasellus* specimens from four stations in Cameroon (Eme et al. 2018). *M. camerounensis* was identified in the two closest stations, while the other two stations, about 70 km geographically distant from each other, proved to harbour two distinct, as yet undescribed species. Recently, a second species, *M. boutini* Poutougnigni, Piscart & Zebaze Togouet, 2021 was described from Douala city. Thus, although knowledge of the stygofauna is still in its infancy in West and Central Africa, the first results show that *Metastenasellus* is indeed present in Benin and Cameroon, and that its species diversity may be much higher than expected.

In the last few years, several stygofauna sampling campaigns in hand-dug wells have taken place in Benin and in Cameroon. They made it possible to accumulate biological material of *Metastenasellus* in dozens of wells in both countries. This material gives us the opportunity to provide a first insight into the species diversity within the genus *Metastenasellus* in these two countries, using a Sanger-based DNA barcoding approach (Hebert et al. 2003).

Materials and methods

Specimens

In Benin, a series of sampling campaigns was organized between 2015 and 2019 in the large Ouémé watershed. A collection of isopod specimens was obtained from a sampling of 169 stations in the Ouémé/ Yéwa basin, 98 of which contained isopods (Fig. 1). Samples were taken in traditional hand-dug, as well as in modern wells lined with casing (as described in BURGÉAP 1981) by means of a modified Cvetkov phreatobiological net (funnel 200 µm mesh size, 150 µm below valve) (Cvetkov 1968; Boutin et al. 2011). For each sampled well, traps baited with a piece of beef were used to collect bottom dwelling animals (Lagnika et al. 2014). Traps were deposited at the bottom of the well for about 24 hours (depending on field constraints). Faunal samples were fixed in ethanol 95% on the spot and were later sorted out at the laboratory of the Zoology Department of the Abomey-Calavi University. Isopod specimens from the same sample were stored in the same vial, preserved in 95% ethanol, and kept in the fridge at 5 °C. Samples were later transferred to the Royal Belgian Institute of Natural Sciences for further processing and were kept at -20 °C in a deep freezer.

In Cameroon, a total of about 150 stations were sampled during field campaigns organized during 2009 and 2019 (Fig. 3). The collection of fauna from the wells was done following the same protocol as in Benin (modified phreatobiological net and baited traps). Baited traps were in place for a period of 10 to 18 h, following the recommendations of Boutin and Boulanouar (1983). The stations were distributed in three major watersheds, namely the Wouri (northwestern Coastal rivers basin), the Sanaga

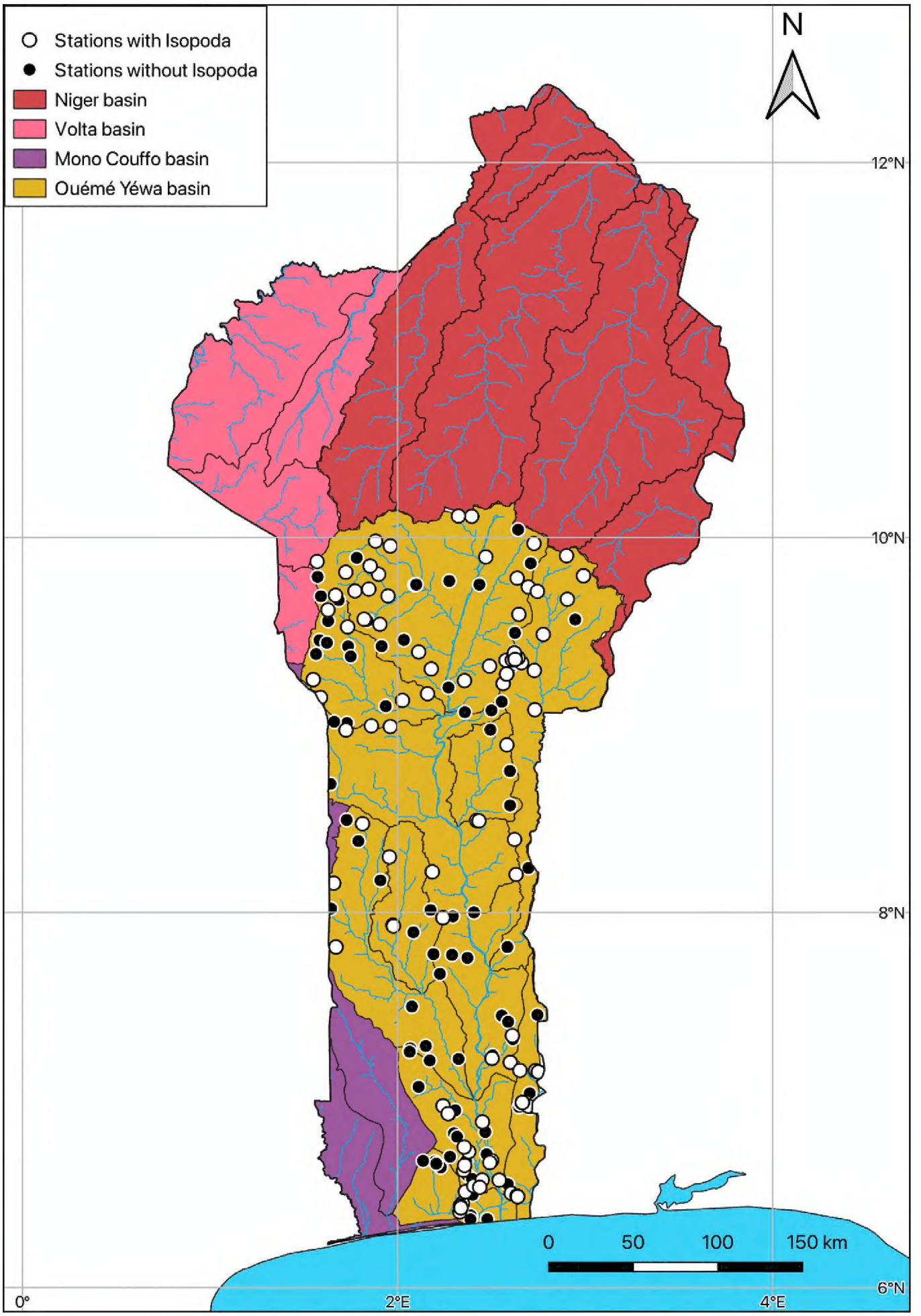

Figure 1. Location of stations sampled in Benin between 2015 and 2019.

and the Nyong (southern Coastal rivers basin). Specimens of the genus *Metastenasellus* were found at 44 stations (Fig. 4), preserved in 95% ethanol, and stored in a freezer at – 4 °C for further molecular processing.

DNA analyses

Specimens

We analyzed a dataset consisting of 89 *Metastenasellus* specimens, of which 57 were collected in Benin, and 32 in Cameroon. One Stenasellidae, *Stenasellus virei*, was used as an outgroup for tree rooting (see below) (Table 1).

The identification of the genus *Metastenasellus* was based on the Magniez (1966) diagnosis, later emended by Magniez (1979) and Zébazé Togouet et al. (2013), and confirmed by Pountougnigni et al. (2021): pleonites 1 and 2 well-developed, dactyli of pereopods 2–7 with one sternal spine, male protopodite of pleopod 1 without a coupling hook, male endopodite of pleopod 2 very voluminous, helicoidal spermatic duct, length ratio of pleonites 1 and 2 to pereonite 7 equal to 2/3 to 1/2.

For Benin, specimens came from a selection of 19 stations chosen so as to have a balanced geographical distribution within the Ouémé basin (Fig. 2). A maximum of three specimens were processed per sample, giving priority to males as their pleopods offer good taxonomic characters for the recognition and further description of new species (Magniez 1966; Magniez and Henry 1983). DNA was extracted from two pereopods, where possible. Specimens were immersed in glycerin for 12 to 24 hours before dissection to soften the appendages made brittle by prolonged stay in ethanol. They were then placed back in 95° ethanol after passing through successive ethanol baths of increasing concentrations. Regarding Cameroon, DNA was extracted and purified from one or two legs (pereopod 1) for large organisms, whereas whole individual were sometimes used for smaller specimens.

DNA extraction and sequencing

DNA was extracted and purified using the Nucleospin Tissue Kit (Macherey-Nagel). Amplification of the COI marker was done by polymerase chain reaction (PCR) followed by Sanger sequencing, using, for the Beninese material, the primers of Folmer et al. (1994) (LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3'; HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and, for the Cameroonian material, their slightly modified version by Astrin and Stüben (2008) (LCO1490-JJ": 5'-CHACWAAYCATAAAGATATYGG-3'; HCO2198-JJ: 5'-AWACTTCVGGRTGVCCAAARAATCA-3'). The PCR mixtures (total volumes of 20 and 50 µl for samples from Benin and Cameroon, respectively) consisted of 1 and 8 µl of DNA extract, final concentrations of 0.4 and 0.16 µM of each primer, 0.03 and 0.025 units/µl of Platinum Taq and DreamTaq DNA Polymerase, 1× reaction buffer, 0.2 mM of dNTP and 1.5 mM of MgCl. The samples were then run in a PCR Thermal Cycler with the following program: an initial denaturation of 3 min at 94 °C followed by 40 and 36 cycles for samples from Benin and Cameroon, respectively of 20 s at 94 °C, 45 s at 50 °C and 60 s at 72 °C and 65 °C. A final extension step of 5 and 2 min at 72 °C and 65 °C was performed in Benin and

Table 1. Specimens included in the study, with their place of deposit (POD – RBINS: Royal Belgian Institute of Natural Sciences; ULB: "Université libre de Bruxelles"), voucher numbers, isolate, MOTUs identified in the ASAP and GMYC analyses, collection data (country, region/department, locality, municipality, latitude, longitude –datum WGS84, station, collection date, collector) and GenBank accession numbers.

Species	POD	Voucher	Isolate	MOTUs	Country	Region/ Department	Municipality; locality	Y coord.	X coord.	Station ID	Collection date	Collector	COI GenBank
Metastenasellus sp.	RBINS	19.304.08	LM423	1	Benin	Plateau	Pobè	7.14983	2.75058	IBT	24-09-2019	M. Lagnika et al	OR797545
	RBINS	19.311.04	LM444	1	Benin	Plateau	Pobè	7.14983	2.75058	IBT	24-09-2019	M. Lagnika et al	OR797559
	RBINS	19.301.10	LM401	2	Benin	Borgou	Ina-CLCAM	9.96883	2.72742	BEN106	1-08-2017	M. Lagnika et al	OR797526
	RBINS	19.301.11	LM402	2	Benin	Borgou	Ina-CLCAM	9.96883	2.72742	BEN106	1-08-2017	M. Lagnika et al	OR797527
	RBINS	19.301.12	LM403	2	Benin	Borgou	Ina-CLCAM	9.96883	2.72742	BEN106	1-08-2017	M. Lagnika et al	OR797528
	RBINS	19.302.02	LM405	2	Benin	Borgou	Gnanhou	9.90350	2.90209	BEN108	2-08-2017	M. Lagnika et al	OR797529
	RBINS	19.302.07	LM410	2	Benin	Borgou	Woria	9.08218	2.73199	BEN143	26-08-2017	M. Lagnika et al	OR797533
	RBINS	19.302.08	LM411	2	Benin	Borgou	Woria	9.08218	2.73199	BEN143	26-08-2017	M. Lagnika et al	OR797534
	RBINS	19.302.09	LM412	2	Benin	Borgou	Woria	9.08218	2.73199	BEN143	26-08-2017	M. Lagnika et al	OR797535
	RBINS	19.302.10	LM413	2	Benin	Borgou	Tchori	9.67146	2.90645	BEN144	27-08-2017	M. Lagnika et al	OR797536
	RBINS	19.302.11	LM414	2	Benin	Borgou	Tchori	9.67146	2.90645	BEN144	27-08-2017	M. Lagnika et al	OR797537
	RBINS	19.302.12	LM415	2	Benin	Borgou	Tchori	9.67146	2.90645	BEN144	27-08-2017	M. Lagnika et al	OR797538
	RBINS	19.311.02	LM442	2	Benin	Borgou	Ina-CLCAM	9.96883	2.72742	BEN106	1-08-2017	M. Lagnika et al	OR797557
	RBINS	19.311.03	LM443	2	Benin	Borgou	Gnanhou	9.90350	2.90209	BEN108	2-08-2017	M. Lagnika et al	OR797558
	RBINS	19.308.11	LM438	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	21-07-2018	M. Lagnika et al	OR797554
	RBINS	19.308.12	LM439	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	21-07-2018	M. Lagnika et al	OR797555
	RBINS	19.311.06	LM446	3	Benin	Borgou	Parakou	9.38486	2.62311	C2	22-09-2019	M. Lagnika et al	OR797560
	RBINS	19.311.07	LM447	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	21-07-2018	M. Lagnika et al	OR797561
	RBINS	19.311.08	LM448	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	21-07-2018	M. Lagnika et al	OR797562
	RBINS	19.311.09	LM449	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	21-07-2018	M. Lagnika et al	OR797563
	RBINS	19.323.02	LM451	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	27-07-2017	M. Lagnika et al	OR797564
	RBINS	19.323.03	LM452	3	Benin	Borgou	Parakou	9.35047	2.62711	C6	27-07-2017	M. Lagnika et al	OR797565
	RBINS	19.304.12	LM427	4	Benin	Plateau	Pobè	6.98711	2.66572	ISH	26-09-2019	M. Lagnika et al	OR797547
	RBINS	19.297.11	LM390	5	Benin	Collines	Agoua N'Tchotche	8.29609	1.95661	BEN052	25-07-2016	M. Lagnika et al	OR797519
	RBINS	19.323.08	LM457	6	Benin	Collines	Tchètti Lema	7.81608	1.67185	BEN072	10-08-2016	M. Lagnika et al	OR797570
	RBINS	19.323.10	LM459	6	Benin	Collines	Tchètti Lema	7.81608	1.67185	BEN072	31-07-2017	M. Lagnika et al	OR797572
	RBINS	19.304.04	LM419	7	Benin	Collines	Tchètti Lema	7.81608	1.67185	BEN072	15-08-2018	M. Lagnika et al	OR797542
	RBINS	19.304.05	LM420	7	Benin	Collines	Tchètti Lema	7.81608	1.67185	BEN072	15-08-2018	M. Lagnika et al	OR797543
	RBINS	19.323.09	LM458	7	Benin	Collines	Tchètti Lema	7.81608	1.67185	BEN072	10-08-2016	M. Lagnika et al	OR797571
	RBINS	19.323.11	LM460	7	Benin	Collines	Tchètti Lema	7.81608	1.67185	BEN072	31-07-2017	M. Lagnika et al	OR797573

Species	POD	Voucher	Isolate	MOTUs	Country	Region/ Department	Municipality; locality	Y coord.	X coord.	Station ID	Collection date	Collector	COI GenBank
Metastenasellus sp.	RBINS	19.301.04	LM395	8	Benin	Donga	Manigri Idiroko	8.97372	1.72553	BEN079	15-08-2016	M. Lagnika et al	OR797522
	RBINS	19.301.07	LM398	8	Benin	Donga	Penessoulou Cs	9.24313	1.55093	BEN086	16-08-2016	M. Lagnika et al	OR797523
	RBINS	19.301.08	LM399	8	Benin	Donga	Penessoulou Cs	9.24313	1.55093	BEN086	16-08-2016	M. Lagnika et al	OR797524
	RBINS	19.301.09	LM400	8	Benin	Donga	Penessoulou Cs	9.24313	1.55093	BEN086	16-08-2016	M. Lagnika et al	OR797525
	RBINS	19.302.04	LM407	8	Benin	Donga	Gangamou	9.84855	1.85416	BEN114	5-08-2017	M. Lagnika et al	OR797530
	RBINS	19.302.05	LM408	8	Benin	Donga	Gangamou	9.84855	1.85416	BEN114	5-08-2017	M. Lagnika et al	OR797531
	RBINS	19.302.06	LM409	8	Benin	Donga	Gangamou	9.84855	1.85416	BEN114	5-08-2017	M. Lagnika et al	OR797532
	RBINS	19.301.01	LM392	9	Benin	Collines	Ouessé CSC	8.48835	2.43420	BEN059	28-07-2016	M. Lagnika et al	OR797520
	RBINS	19.301.03	LM394	9	Benin	Collines	Ouessé CSC	8.48835	2.43420	BEN059	28-07-2016	M. Lagnika et al	OR797521
	RBINS	19.323.04	LM453	9	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	2-08-2015	M. Lagnika et al	OR797566
	RBINS	19.308.05	LM432	10	Benin	Ouémé	Porto-Novo	6.48492	2.64036	LB	29-06-2019	M. Lagnika et al	OR797551
	RBINS	19.308.06	LM433	10	Benin	Ouémé	Porto-Novo	6.48492	2.64036	LB	29-06-2019	M. Lagnika et al	OR797552
	RBINS	19.308.08	LM435	10	Benin	Ouémé	Porto-Novo	6.48856	2.63744	AH	29-09-2019	M. Lagnika et al	OR797553
	RBINS	19.309.13	LM440	10	Benin	Ouémé	Porto-Novo	6.48450	2.64008	AR	23-06-2019	M. Lagnika et al	OR797556
				10	Benin	Ouémé	Porto-Novo	6.48492	2.64036	LB		M. Lagnika et al	KY623773
				10	Benin	Ouémé	Porto-Novo	6.48492	2.64036	LB		M. Lagnika et al	KY623774
	RBINS	19.304.01	LM416	11	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	9-06-2018	M. Lagnika et al	OR797539
	RBINS	19.304.02	LM417	11	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	9-06-2018	M. Lagnika et al	OR797540
	RBINS	19.304.03	LM418	11	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	9-06-2018	M. Lagnika et al	OR797541
	RBINS	19.304.07	LM422	11	Benin	Plateau	Pobè	7.14983	2.75058	IBT	24-09-2019	M. Lagnika et al	OR797544
	RBINS	19.304.09	LM424	11	Benin	Plateau	Pobè	7.14983	2.75058	IBT	24-09-2019	M. Lagnika et al	OR797546
	RBINS	19.308.01	LM428	11	Benin	Plateau	Pobè	7.15653	2.73597	TW	24-09-2019	M. Lagnika et al	OR797548
	RBINS	19.308.03	LM430	11	Benin	Plateau	Pobè	7.15653	2.73597	TW	24-09-2019	M. Lagnika et al	OR797549
	RBINS	19.308.04	LM431	11	Benin	Plateau	Pobè	7.15653	2.73597	TW	24-09-2019	M. Lagnika et al	OR797550
	RBINS	19.323.05	LM454	11	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	27-01-2017	M. Lagnika et al	OR797567
	RBINS	19.323.06	LM455	11	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	27-01-2017	M. Lagnika et al	OR797568
	RBINS	19.323.07	LM456	11	Benin	Plateau	Oke-Ola	7.22220	2.50389	BEN031	27-01-2017	M. Lagnika et al	OR797569
	ULB	RK19_14	RK19_14	12	Cameroon	Littoral	Mbanga; Mb1	4.29030	9.34022		11-2018	R. Kayo	OR791025
	ULB	RK19_01	RK19_01	12	Cameroon	Littoral	Mbanga; Mb1	4.29030	9.34022		10-2018	R. Kayo	OR791023
	ULB	RK19_88	RK19_88	12	Cameroon	Southwest	Muyuka; Owé 3S	4.17264	9.23050		11-2017	R. Kayo & Chinche	OR791041
				12	Cameroon	Littoral	Moungo; Loum 1	4.43100	9.43300				KY623775
	ULB	RK19_34	RK19_34	13	Cameroon	West	Foumbam; Fbt6	5.29550	10.3718		11-2018	R. Kayo & Farikou	OR791036
				14	Cameroon	Littoral	Douala; Makepe 2	4.06800	9.72100				KY623776

Species	POD	Voucher	Isolate	MOTUs	Country	Region/ Department	Municipality; locality	Y coord.	X coord.	Station ID	Collection date	Collector	COI GenBank
Metastenasellus				15	Cameroon	Littoral	Douala; P1	4.12411	9.82686				OL514108
boutini				15	Cameroon	Littoral	Douala; P3	4.12144	9.82833				OL514109
				15	Cameroon	Littoral	Douala; P4	4.12111	9.82817				OL514110
				15	Cameroon	Littoral	Douala; P10	4.11908	9.82592				OL514111
				15	Cameroon	Littoral	Douala; P3	4.12144	9.82833				OL514112
Metastenasellus sp.	ULB	RK19_15	RK19_15	16	Cameroon	Central	Nkoteng; Addic	4.43160	12.09130		11-2018	R. Kayo & Mdejo	OR791026
	ULB	RK19_19	RK19_19	17	Cameroon	Southwest	Muyuka; OS2	4.17542	9.22399		11-2017	R. Kayo & Chinche	OR791030
	ULB	RK19_18	RK19_18	18	Cameroon	Southwest	Tiko; TW10	4.04531	9.22081		02-2017	R. Kayo & Chinche	OR791029
	ULB	RK19_32	RK19_32	18	Cameroon	Southwest	Tiko; TW3	4.04543	9.21073		08-2017	R. Kayo & Chinche	OR791035
	ULB	RK19_23	RK19_23	19	Cameroon	West	Dcshang; Pb7C3	5.27080	10.04340		03-2018	R. Kayo & Madiesse	OR791032
	ULB	RK19_17	RK19_17	20	Cameroon	West	Dschang; PP5C2MKN	5.56420	10.31230		03-2018	R. Kayo & Madiesse	OR791028
	ULB	RK19_25	RK19_25	21	Cameroon	West	Dschang; Pb6C2MKN	5.56060	10.04670		02-2018	R. Kayo & Madiesse	OR791033
	ULB	RK19_30	RK19_30	21	Cameroon	West	Dschang; Pd9C1MKN	5.34650	10.08620		04-2018	R. Kayo & Madiesse	OR791034
	ULB	RK19_37	RK19_37	21	Cameroon	West	Dschang; Pd6C1	5.42670	10.07910			R. Kayo & Madiesse	OR791038
	ULB	RK19_22	RK19_22	22	Cameroon	Central	Nkoteng; Essoboutou	4.33150	12.08140		08-2016	R. Kayo & Mdejo	OR791031
	ULB	RK19_35	RK19_35	22	Cameroon	Central	Nkoteng; Camp Nouveau	4.45120	12.08190		10-2016	R. Kayo & Mdejo	OR791037
	ULB	RK19_02	RK19_02	23	Cameroon	Central	Yaoundé; Olembé	3.41247	11.26854		06-2017	R. Kayo & Tayou	OR791024
	ULB	RK19_16	RK19_16	23	Cameroon	Central	Olembé; Olembé3	3.49720	11.33966		11-2018	R. Kayo & Tayou	OR791027
	ULB	RK19_47	RK19_47	23	Cameroon	Central	Yaoundé; PM3	3.55212	11.24516		08-2017	R. Kayo & Tayou	OR791039
	ULB	RK19_48	RK19_48	23	Cameroon	Central	Yaoundé; PM5	3.50010	11.26853		06-2017	R. Kayo & Tayou	OR791040
	ULB	RK19_93	RK19_93	23	Cameroon	Central	Yaoundé; Olembé III	3.49720	11.33966		05-2017	R. Kayo & Tayou	OR791042
	ULB	RK19_95	RK19_95	23	Cameroon	Central	Yaoundé; Messassi 1	3.41612	11.26231		05-2017	R. Kayo & Tayou	OR791043
Metastenasellus				23	Cameroon	Central	Yaoundé; Emana	3.91900	11.52000				KY623769
camerounensis				23	Cameroon	Central	Mefou-et-Akono; Ebogo 1	3.56800	11.35400				KY623772
				23	Cameroon	Central	Yaoundé; Emana	3.91900	11.52000				KY623770
				23	Cameroon	Central	Yaoundé; Emana	3.91900	11.52000				KY623771
Stenasellus virei					Spain	Guadalajara	Trillo	40.69067	-2.58333				JQ921669

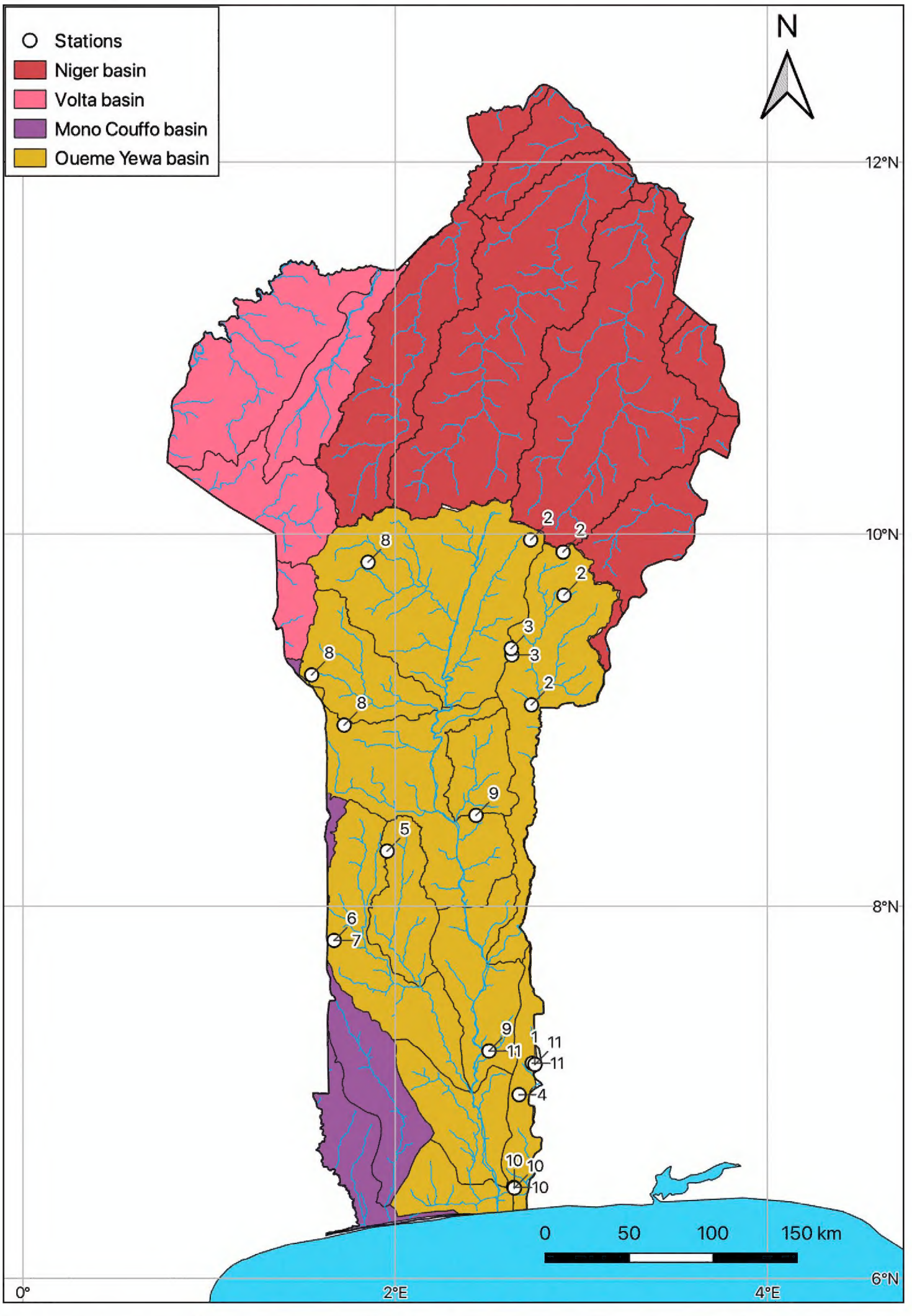

Figure 2. Location of the 19 stations in the Ouémé basin (Benin) from which 57 *Metastenasellus* specimens were selected for DNA taxonomy. The numbers connected to the station symbols correspond to the numbering of the different MOTUs identified in this study.

Cameroon, respectively. PCR products were checked after migration by electrophoresis in a 1.2% agarose gel and, for Benin, were purified using the ExoSAP procedure (Exonuclease I– Shrimp Alkaline Phosphatase from ThermoFisher, USA). Successful amplicons were sequenced bidirectionally at Macrogen Europe BV (Amsterdam, The Netherlands) and at Laboratoire Genoscreen (France) for samples from Benin and Cameroon, respectively.

The resulting sequences were assembled and cleaned using CodonCode Aligner v.8.0.2 (CodonCode Corporation) and Sequencher v.4.1.4 (Gene Codes Corporation), and compared to sequences already published in international databases using NCBI BLAST (NCBI Resource Coordinators 2016). For Benin, 76 contigs were obtained from the 81 DNA extracts and, after elimination of poor-quality sequences, a final selection of 57 contigs (specimens from 19 different stations) was retained. This dataset was supplemented by 2 additional sequences of *Metastenasellus* made available on GenBank by Eme et al. (2018), from specimens collected in Benin by one of us (ML).

For Cameroon, the 29 contigs obtained in this study were completed by 4 sequences made available by Eme et al. (2018) in GenBank, from specimens collected by one of us (RTK), as well as 5 sequences of *M. boutini* published by Pountougnigni et al. (2021) (Table 1).

Molecular phylogeny

A phylogenetic tree was inferred by maximum likelihood using IQ-TREE v. 2.2.0 for macOS (Nguyen et al. 2015), with the best-fit model, TN+F+I+G4, automatically selected by the software, according to the Bayesian Information Criterion, via ModelFinder (Kalyaanamoorthy et al. 2017), as well as optimisation of its parameters, and data partitioned according to codon position. Branch support was obtained with the ultrafast bootstrap with 1000 replicates (Hoang et al. 2018).

Distance analysis

Uncorrected pairwise genetic distances were calculated using MEGA 11 (Tamura et al. 2021), after trivial alignment of COI sequences facilitated using the MUSCLE algorithm (Edgar 2004). Genetic distances were calculated between sequences, and between and within MOTUs (Molecular Operational Taxonomic Units) as identified by the single-locus approaches ASAP and GMYC (see below).

Single-locus species delimitation

Species were delineated following complementary approaches (Dellicour and Flot 2015): a distance-based method, ASAP ("Assembling Species by Automatic Partitioning") (Puillandre et al. 2021), and two tree-based methods, the "General Mixed Yule Coalescent" models, GMYC (Pons et al. 2006), and the "Poisson Tree Processes" (PTP) method (Zhang et al. 2013).

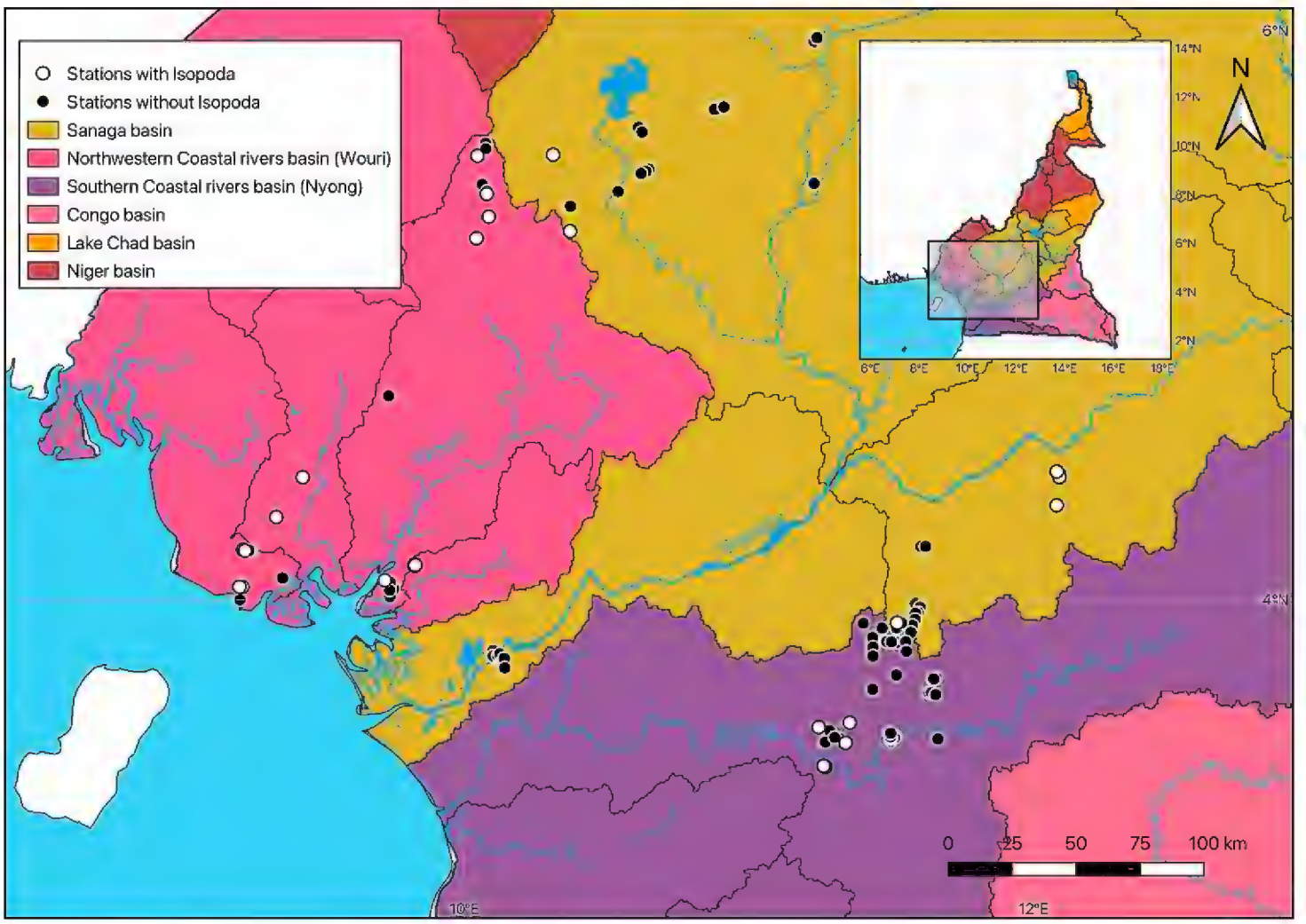

Figure 3. Location of stations sampled in Cameroon between 2009 and 2019.

ASAP was run using p-distances as well as both the Jukes-Cantor (JC69) and the Kimura 2-parameter (K80) substitution models to compute the distances, in order to investigate the possible impact of different distance models on the partitioning. Analyses were performed on the dedicated public web server (https://bioinfo.mnhn.fr/abi/public/asap/).

For the GMYC analysis, transition between inter- and intra-species branching rates were estimated on an ultrametric tree reconstructed using BEAST v2.7.2 (Bouckaert et al. 2019) (without time calibration). The Bayesian inference of phylogeny was performed using the TN+F+I nucleotide substitution model, as identified in the IQ-TREE analysis, with four gamma categories estimated by the software, a strict molecular clock model and the Yule prior with default parameters. The analysis was run with a Markov Chain Monte Carlo (MCMC) length of 10 million. The first 10% of the trees were discarded as "burn-in" and marginal posterior estimates were checked using Tracer v1.7.2. (Rambaut et al. 2021). The maximum credibility tree obtained from the BEAST analysis was imported in R v4.2.2 and submitted to the gmyc function available in the R package splits v1.0–20 (Ezard et al. 2021).

PTP analyses were performed using multi-rate PTP (mPTP), which, unlike PTP, takes into account differences in intraspecific variation, due to the evolutionary history or sampling of each species. mPTP is presented as an improvement on the single-rate model PTP (Zhang et al. 2013), making possible to obtain more accurate estimates than the latter (Kapli et al. 2017). bPTP, the Bayesian implementation of the single-rate PTP, was also used to check the congruence of results with mPTP. Unlike GMYC,

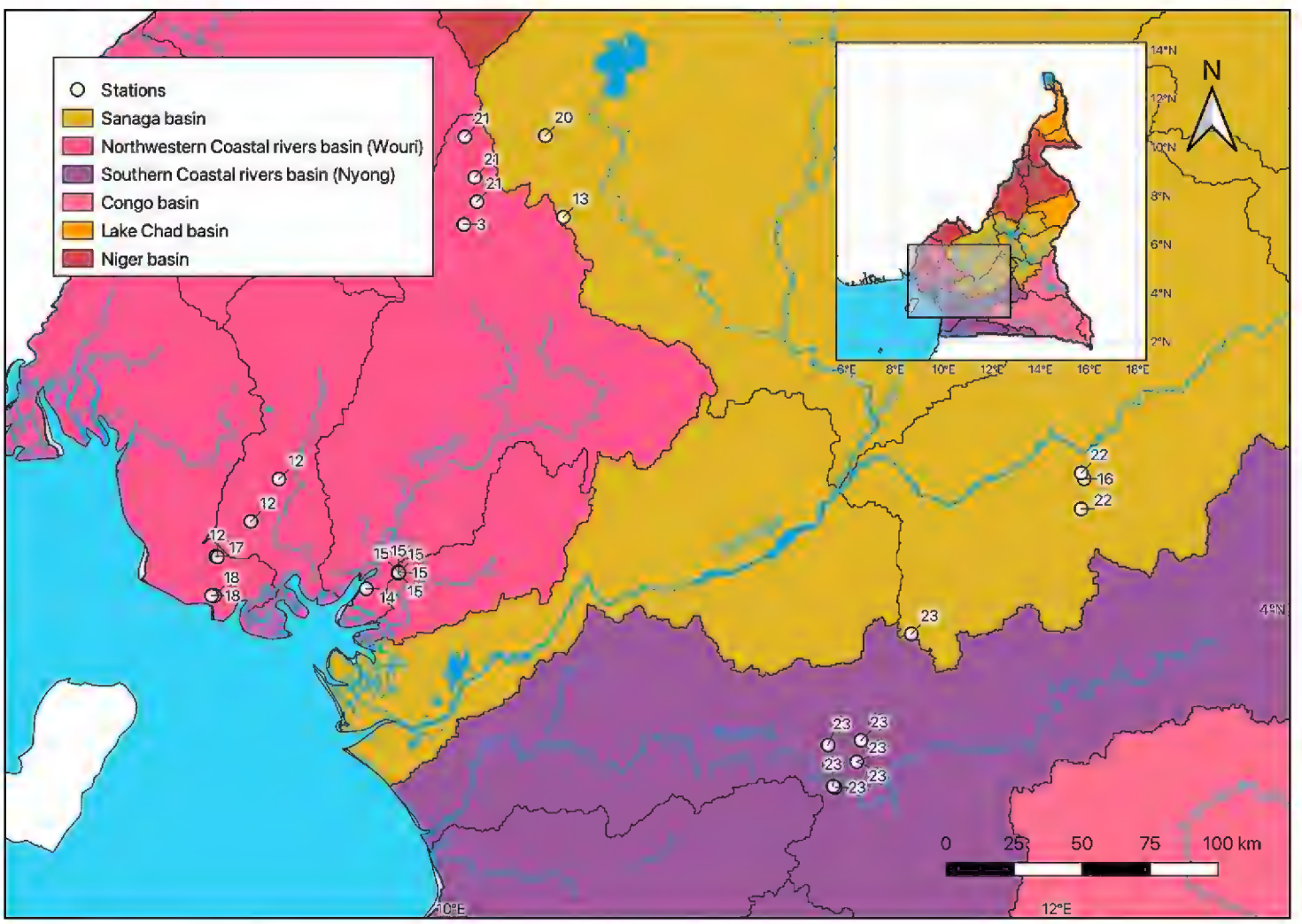

Figure 4. Location of the 44 stations where *Metastenasellus* specimens were found in Cameroon and studied for DNA taxonomy (white dots). The numbers connected to the station symbols correspond to the numbering of the different MOTUs identified in this study.

PTP analyses do not require an ultrametric input tree, which is a potentially error-prone process (Zhang et al. 2013). Therefore, the phylogenetic tree produced by the I-QTREE analysis was used as the input tree for all analyses based on the PTP model. bPTP analysis was performed on the bPTP web server (https://species.h-its.org/). The stand-alone version of mPTP was preferred to its web implementation because certain functionalities are not available in the web service (https://mptp.h-its.org/), in particular the computation of support values for each clade, using MCMC. The last release of the pre-compiled macOS binary (mPTP 0.2.4) was downloaded from GitHub (https://github.com/Pas-Kapli/mptp).

Results

Species delimitation

Both ASAP or GMYC delineated 23 similar MOTUs that corresponded to singletons or strongly supported clades in the ML tree (BV: 99–100) (Fig. 5). ASAP analyses consistently suggested the same partitioning into 23 different MOTUs, regardless of how the distances were estimated (p-distances, JC69, K80), 11 MOTUs in Benin and 12 MOTUs in Cameroon.

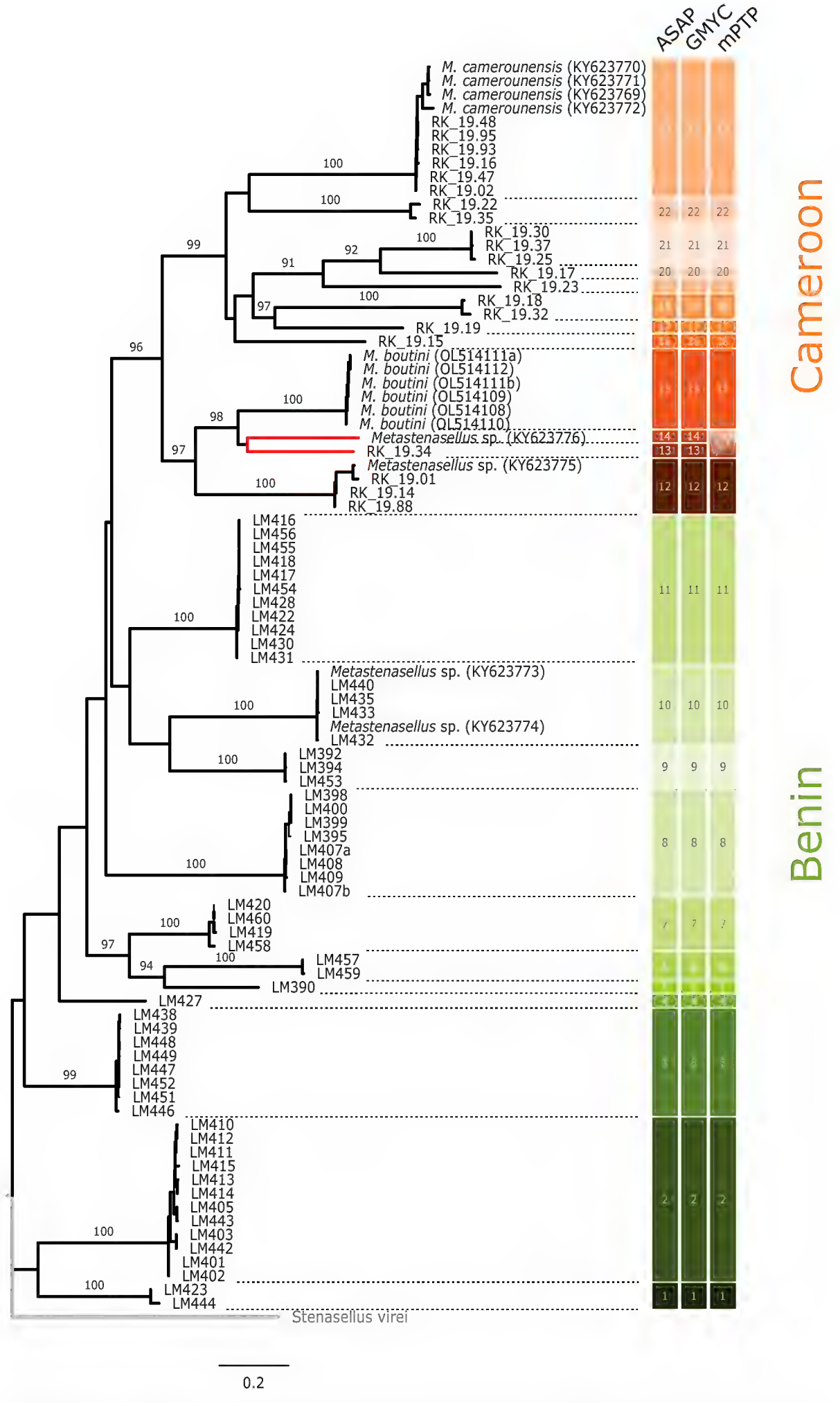

Figure 5. Molecular phylogeny constructed using the maximum likelihood method and COI gene fragment of *Metastenasellus* specimens from Benin and Cameroon. Partitions at the right side of the figure represent the results of the species delimitation analyses with single-locus methods (ASAP, GMYC, bPTP). Numbers at nodes are ultrafast bootstrap values (BV). Nodes were considered as supported if BVs were higher or equal to 90 (Hoang et al. 2018). For the sake of clarity, BVs are not shown within MOTUs delimited by ASAP and GMYC.

mPTP delineated 22 MOTUs, the same as ASAP and GMYC except for MOTUs 13 and 14 which were lumped, albeit separated by long branches on the tree (in red; Fig. 5), and uncorrected pairwise distances as high as 19.2% (Table 2; see below). However, this delimitation was the only one to receive no support (0.2), unlike all the others which received support values ranging from 0.64 to 1.00.

In contrast, bPTP delineated 26 MOTUs. Compared to others approaches, MOTU 1 was split into two singleton MOTUs, and MOTU 12 into two singleton MOTUs (RK_19.88), (RK_19.14) and a duo MOTU (KY623775, RK_19.01). Unlike mPTP, MOTUs 13 and 14 remained distinct.

Distance analysis

Uncorrected pairwise distances between specimens ranged between 0.0 and 28.6%. Considering the 23 MOTUs defined according to the results of the species delimitation analyses performed with ASAP and GMYC (Fig. 5), the maximal distances within MOTUs varied between 0.2% (MOTU 9, MOTU 10) and 3.5% (MOTU 12, MOTU 22) while the mean distances between MOTUs varied between 17.1% (M5, M7) and 27.5% (M8, M18; M9, M23; M10, M22; M12, MM18) (Table 2).

Discussion

Species delimitation

The main objective of this study was to provide a first insight into the species diversity within the genus *Metastenasellus* in Benin and Cameroon. For this, an accurate delimitation of species is not yet required, although it will be desirable in the future. In this respect, the use of a single-locus approach as a first step in a species delimitation is justified, despite its well-known weaknesses (Leliaert et al. 2014). Even if a single locus may not follow the history of the species, due to introgression and incomplete lineage sorting (Puillandre et al. 2021), it nevertheless provides a first overview on the species-level diversity within a group.

Whether ASAP, GMYC or PTP, all these methods provide congruent results in suggesting about 23 highly divergent lineages, once the probably misleading lumping of MOTUs M13 and M14 in the mPTP analysis has been excluded (MOTUs M13 and M14 are separated by p-distances as high as 19.1% and appear to be joined by long branches in the ML tree, making their lumping into one hypothetical species questionable). In contrast, the bPTP approach provided some species hypotheses that are highly unlikely, e.g. splitting MOTUs with p-distances values as low as 2.1% (M1) and 3.5% (M12).

The performance of each method is variable and subject to its own errors, resulting in either oversplitting or overlumping (Dellicour and Flot 2018), and the GMYC approach is known to belong to the first category (Puillandre et al. 2021). In this respect, it is reassuring to see that ASAP, GMYC and, to a lesser extent PTP, all yielded

Table 2. Estimates of evolutionary divergence over sequence pairs between and within MOTUs (M) identified with the ASAP analysis (average uncorrected pairwise distances in per cent).

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	M21	M22	M23
M1	2.1																						
M2	20.0	2.0																					
M3	19.1	18.9	0.6																				
M4	18.5	21.2	18.8	n/c																			
M5	20.1	19.7	18.4	20.8	n/c																		
M6	21.5	21.3	22.3	23.3	20.4	0.3																	
M7	21.8	19.2	18.4	20.1	17.1	21.5	1.4																
M8	21.5	22.1	21.5	19.9	22.9	23.4	21.8	1.1															
M9	22.5	23.7	23.6	23.0	22.9	25.4	21.3	23.0	0.2														
M10	24.8	23.5	23.9	22.5	23.8	23.2	23.7	23.3	20.9	0.2													
M11	20.5	20.6	20.1	20.5	19.7	22.4	19.4	22.6	21.8	20.4	0.3												
M12	24.6	24.0	25.6	25.0	26.2	25.6	24.7	25.6	25.0	25.6	23.8	3.5											
M13	23.7	23.3	21.2	21.0	24.5	26.1	25.0	21.7	23.3	24.4	22.1	22.8	n/c										
M14	23.5	22.6	25.1	22.4	22.8	23.0	23.1	22.6	24.3	23.1	22.4	23.1	19.1	n/c									
M15	23.2	23.7	21.5	22.5	23.0	24.5	22.3	24.1	22.7	23.9	19.7	22.1	19.3	19.1	0.5								
M16	21.7	23.9	23.1	23.3	21.9	24.6	22.2	24.3	23.7	26.1	24.0	24.4	22.2	23.3	25.0	n/c							
M17	24.2	23.3	23.4	22.3	22.3	24.6	24.5	23.7	25.7	25.5	22.8	24.9	24.2	24.2	22.5	21.1	n/c						
M18	23.3	23.3	22.5	25.1	24.6	26.1	22.9	27.5	24.7	26.0	25.6	27.5	25.8	23.9	25.1	21.6	21.7	2.4					
M19	26.8	24.2	24.4	24.3	25.7	25.1	25.5	25.4	25.6	23.7	23.7	27.3	25.7	23.8	25.4	22.6	24.8	24.5	n/c				
M20	23.2	24.3	23.0	22.9	22.0	25.7	22.1	24.7	23.9	26.0	22.5	26.3	21.9	24.1	21.9	19.9	22.2	23.9	21.6	n/c			
M21	22.3	23.9	22.7	21.9	22.5	24.1	22.5	22.6	26.3	24.2	22.4	24.9	23.4	25.4	24.8	21.1	23.7	25.6	19.9	19.6	0.8		
M22	26.1	26.8	24.7	22.6	25.8	24.9	24.7	23.1	27.2	27.5	23.3	25.0	24.1	24.9	26.7	21.1	23.2	24.5	25.0	24.5	23.8	3.5	
M23	25.7	26.4	26.9	25.4	26.1	27.1	26.9	24.8	27.5	25.7	26.7	23.6	24.0	26.1	24.5	23.6	23.7	25.1	24.6	25.0	25.6	24.3	2.5

congruent results. This congruence can be interpreted as compelling evidence for the reliability of the outcomes obtained. With regard to the PTP approach, it is interesting to note that mPTP produces results closer to those of ASAP and GMYC than bPTP. This is consistent with the observation that mPTP is superior to PTP in producing delimitations more congruent with taxonomy (Kapli et al. 2017).

Although it is not advisable to consider MOTUs as distinct species on the basis of the mitochondrial COI alone, without at least including a nuclear marker (Dellicour and Flot 2018), several evidences suggest that most, if not all MOTUs identified by the congruence of the different methods used herein corresponds to a valid species.

The first piece of evidence is the particularly high mean p-distances between MOTUs (17.1 to 27.5%). Based on a dataset including a wide taxonomic coverage of Crustacea, Lefébure et al. (2006) have suggested that two monophyletic groups divergent by more than 0.16 substitution per site in the COI gene, as measured by patristic distances, have a strong probability to belong to different species. Patristic distances are defined as the amount of divergence since two taxa shared a common ancestor, i.e., the path-length distance between the two taxa along a phylogenetic tree. Later, Morvan et al. (2013) showed that the threshold method of Lefébure et al. (2006) applied to Aselloidea remained relevant. In this study, mean p-distances between *Metastenasellus* MOTUs are well above this threshold. This observation makes even more sense given that patristic distances are necessarily higher than p-distances because, unlike the latter, they take account of multiple substitutions. It should also be noted that the separation between the lineages must probably be ancient, as suggested by the particularly large distances between MOTUs, which would make the biases usually associated with the use of a single locus for species delimitation (such as introgression or incomplete sorting of lineages), all the more unlikely. It should be noted, however, that Raupach et al. (2022) have recently shown the existence of surprisingly high genetic divergences in the DNA barcode fragment (COI) within some woodlouse species, which it seems difficult to attribute to the existence of cryptic species, an observation that requires, however, future confirmation with nuclear markers.

Second evidence is the observation that two morphologically distinct species, *M. boutini* (M15) and *M. camerounensis* (M23), are separated by p-distances of 24.5%, i.e., a mean interspecific distance of the same order of magnitude as the distances between most other MOTUs (Table 2). An ongoing morphological study also shows that specimens from the same faunistic samples as MOTUs 9, 10 and 11 have clearly distinct male pleopods 2, confirming that these MOTUs correspond to distinct species (Lagnika, pers. comm.). These three MOTUs are separated by p-distances ranging from 20.4 to 24.8% (Table 2).

Third piece of evidence is the coexistence of two MOTUs in the same station, at the same time (BEN072: M6, M7; IBT: M1, M11) indicating that these MOTUs are separately evolving lineages, in other words potential distinct species according to the de Queiroz's species concept (de Queiroz 2007). However, it is important to keep in mind that divergent mitogenomes found in sympatry are not always associated with divergent nuclear sequences (Martinsson et al. 2020) and may coexist in a single species (Giska et al. 2015).

Diversity and endemism in *Metastenasellus*

A high level of species diversity in the genus *Metastenasellus* in Benin and Cameroon, with about 23 potential distinct species, is all the more remarkable given that, although this is the first significant sampling effort in these two countries, it is still very limited given the geographic area of these countries. However, it is not really a surprise given the recent realisation that the aquatic groundwater environment harbours rich macrobiological diversity, with a high level of endemism and numerous relict species (Gibert et al. 1994; Dole-Olivier et al. 2005; Gibert et al. 2009; Halse et al. 2014; Borko et al. 2021).

Although this study focused on a limited number of *Metastenasellus* stations, the distribution maps (Figs 2, 4) show a narrow geographical distribution of all MOTUs, suggesting high levels of endemism. The coexistence of two MOTUs in the same station remains the exception, but this may result from the limited sampling effort. In groundwater habitats, exceptional levels of endemism are generally assumed to be caused by strong hydrogeographical isolation, resulting in vicariance, and low dispersal abilities of their inhabitants (Gibert et al. 2009; Iepure et al. 2021). Following an exhaustive analysis of the literature on Stenasellidae in Africa, Pountougnigni et al. (2021) had already observed that the known distribution of this isopod family on this continent was very patchy, with most species known only from their type localities. It is generally accepted that hyporheic habitats of rivers can act as dispersal corridors for subterranean aquatic animals (Stanford and Ward 1993; Malard et al. 2023). In Benin, although the Ouémé river could have played this role, no MOTU from the upper part of the Ouémé basin was observed in the lower part of the basin, which suggests significant faunal isolation.

In Europe, Trontelj et al. (2009) suggested that macro-stygobiotic species showing range sizes over 200 km were most likely an assemblage of cryptic species with much smaller geographic ranges. Our data do not contradict this observation for this region of Africa as well. If so, we can expect future studies to reveal levels of diversity of stygobiotic isopods as high as those documented in other parts of the world. In Western Europe alone, Morvan et al. (2013) identified about 150 species and 12 genera of obligate groundwater Aselloidea (values corrected excluding groundwater species from USA, Japan, Lebanon and Mexico). This observation alone shows the extent to which the diversity of Aselloidea in Africa is probably unknown and remains to be discovered.

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